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# Safety assessment of an etidronate in a sodium hypochlorite solution: randomized double-blind trial

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Abstract: Aim To assess whether Dual Rinse HEDP, an etidronate that can be combined with NaOCl to create an endodontic irrigating solution containing both hypochlorite and a chelator in the form of 1-hydroxyethane 1,1-diphosphonic acid (HEDP), alters the clinical efficacy of NaOCl or adds any untoward clinical effects. Methodology In this randomized controlled double-blind single-centre trial, a pure NaOCl solution was compared to a HEDP-containing counterpart regarding antimicrobial efficacy, postoperative pain, and the host response by means of changes in MMP-9 levels in periapical fluid. Sixty patients presenting with asymptomatic apical periodontitis (one tooth each) were randomly divided into two groups (N = 30) based on irrigation regime. Pre- and post-treatment microbial aerobic and anaerobic cultures and MMP-9/total protein (TP) periapical fluid samples were collected. Postoperative pain levels were assessed 24 h after treatment. Categorical data were compared between groups using the Fisher's exact test, continuous data using the Wilcoxon signed-rank test,  = 0.05. Results Irrigation with pure NaOCl rendered 40% canals free of culturable microorganisms, compared to 50% with the NaOCl/HEDP mixture (P = 0.60). As assessed by matrix-assisted laser desorption/ionization-time-of-flight analysis (MALDI-TOF), no apparent selection of aerobic or anaerobic taxa occurred in either group. One patient in the NaOCl group experienced moderate pain, whilst two patients in the NaOCl/HEDP group experienced mild postoperative pain. MMP-9/TP levels in periapical fluid declined significantly ($P < 0.001$) after 1 week with no medication in the root canal, without significant difference between treatment groups ($P > 0.05$). Conclusions This trial found no influence of HEDP on clinical NaOCl effects.

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# Efficacy and safety of etidronate in a sodium hypochlorite solution: randomized double-blind trial

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**Keywords:** Endodontics, Inflammation, Bacteria, Evidence-based dentistry/health care, Matrix metalloproteinases (MMPs), Clinical outcomes

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## Abstract

**Aim** To assess whether Dual Rinse HEDP, an etidronate that can be combined with NaOCl to create an endodontic irrigating solution containing both hypochlorite and a chelator in the form of 1-hydroxyethane 1,1-diphosphonic acid (HEDP), alters the clinical efficacy of NaOCl or adds any untoward clinical effects.

**Methodology** In this randomized controlled double-blind single-center trial, a pure NaOCl solution was compared to a HEDP-containing counterpart regarding antimicrobial efficacy, postoperative pain, and the host response by means of changes in MMP-9 levels in periapical fluid. Sixty patients presenting with asymptomatic apical periodontitis (one tooth each) were randomly divided into two groups ($N$ = 30) based on irrigation regime. Pre- and post-treatment microbial aerobic and anaerobic cultures and MMP-9/total protein (TP) periapical fluid samples were collected. Postoperative pain levels were assessed 24 h after treatment. Categorical data was compared between groups using Fisher's exact test, continuous data using Wilcoxon signed-rank test, $\alpha = 0.05$.

**Results** Irrigation with pure NaOCl rendered 40% canals free of culturable microorganisms, compared to 50% with the NaOCl/HEDP mixture ($P = 0.60$). As assessed by Matrix Assisted Laser Desorption Ionization Time-of-Flight analysis (MALDI-TOF), no apparent selection of aerobic or anaerobic taxa occurred in either group. One patient in the NaOCl group experienced moderate pain, while two patients in the NaOCl/HEDP group experienced mild postoperative pain. MMP-9/TP levels in periapical fluid declined significantly ($P < 0.001$) after one week with no medication in the root canal, without significant difference between treatment groups ($P > 0.05$).

**Conclusions** This trial found no influence of HEDP on clinical NaOCl effects.

## Introduction

Apical periodontitis is characterized by a complex interplay between microbial tissue invasion and host defense (Hahn & Liewehr 2007). The aim of root canal treatment in teeth with this condition is to eliminate microbial biofilm and by-products from the root canal system to promote apical healing. Cleaning and shaping the root canals using endodontic instruments and irrigating solutions plays an essential role in this context (Byström & Sundqvist 1985). Sodium hypochlorite (NaOCl) solutions are the most widely used irrigants for root canal disinfection due to their unique effectiveness against biofilms and their ability to dissolve organic matter (Zehnder 2006). However, NaOCl solutions lack the ability to remove the inorganic aspects of the smear layer and accumulated hard tissue debris formed during mechanical instrumentation (Paqué *et al.* 2011). Therefore, alternating application of the sequestering (chelating) agent ethylenediaminetetraacetic acid (EDTA) and NaOCl is commonly advocated (Baumgartner & Mader 1987). However, EDTA has been found to eradicate active chlorine when combined with NaOCl (Grawehr *et al.* 2003), and may erode dentine if used overzealously (Calt & Serper 2002). EDTA is therefore advocated as a final irrigant before root filling or placing the interim dressing.

Etidronic acid (more precisely: 1-hydroxyethane 1,1-diphosphonic acid or HEDP) is a mild chelator that is compatible with NaOCl in the short term (Zehnder *et al.* 2005). It can be used in combination with a NaOCl solution, a concept that has been termed “continuous chelation” (Neelakantan *et al.* 2012). The main clinical advantage of a combined use of HEDP and NaOCl for root canal irrigation is the simplicity and time-saving application compared to alternately flushing root canals with two irrigants. However, until recently, no clinically approved material based on this chemistry was available, and all studies were based on laboratory experiments. These investigations have suggested that a combined NaOCl/HEDP solution could have various beneficial effects compared to a pure NaOCl solution, e.g. the prevention of a smear layer (Lottanti *et al.* 2009) and the reduction of hard tissue debris accumulation during root canal instrumentation (Paqué *et al.* 2012). In addition, a possible reduction of torsional load on rotary instruments has been described (Boessler *et al.* 2007). It has also been shown that HEDP in fresh mixtures with NaOCl does not reduce the antibacterial effect of the latter (Arias-Moliz *et al.* 2014). It may be that HEDP can improve the disinfection of NaOCl in the presence of a smear layer or hard tissue debris (Morago *et al.* 2016).

With the lack of clinical investigations, however, it is not known whether HEDP would reduce the effectiveness of NaOCl in the clinical situation, or whether any unintended side effects could occur. Consequently, before any superiority studies are performed, a clinical safety assessment is required. It is conceivable that HEDP, which does react slowly with the NaOCl in a combined irrigant, could reduce its antimicrobial effectiveness (Zollinger *et al.* 2017). Moreover, HEDP, by means of its reduction of debris accumulation in apical parts of the root canal, could induce over-irrigation and thus increase postoperative pain and/or inflammatory changes in periapical tissues (Gondim *et al.* 2010). Dual Rinse HEDP (Medcem, GmbH, Weinfelden, Switzerland) is the first HEDP product approved for use in the root canal. It comes in a capsule containing 0.9 g of etidronate powder, which should be mixed immediately with 10 mL of a NaOCl solution of choice directly before treatment, resulting in a combined irrigant containing both active chlorine and approximately 9% HEDP (Zollinger *et al.* 2017).

In the present safety trial on adult patients presenting with teeth affected by primary asymptomatic apical periodontitis, root canals were either irrigated with a pure 2.5% NaOCl solution or a combined 2.5% NaOCl/ 9% Dual Rinse HEDP solution during the cleaning and shaping procedure. EDTA was not used in the control arm of this trial to not divert from the core question, which was: “are there any untoward effects added to the NaOCl by combining it with an etidronate?” The evaluated outcomes were: (a) the percentage of root canals rendered free of culturable bacteria; (b) postoperative pain levels, and (c) host response assessed by the levels of MMP-9 (neutrophil gelatinase) in the periapical fluid.

## Materials and methods

### Study design

This was a randomized controlled double-blind single-centre clinical trial with two parallel experimental arms. The trial was approved by the institutional ethics committee and registered at Clinical Trials Registry (CTRI/2017/08/009493). All patients were informed regarding the benefits, risks, and alternative treatment choices before enrollment in the trial. They were also informed that not participating in this study had no consequences regarding their treatment whatsoever. Informed consent was obtained from all patients. The study was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki, and the Institutional ethical committee, The CONSORT guidelines (2010) for randomized trials were followed.

**Inclusion and exclusion criteria**

The inclusion criteria were patients aged 18 years or above attending the Department of Conservative Dentistry and Endodontics, presenting with a tooth with a pulpal diagnosis of necrosis and an apical diagnosis of asymptomatic apical periodontitis (American Association of Endodontists 2009). The diagnosis was established according to the patient's history, clinical inspection, palpation, tenderness to percussion, pulpal sensitivity testing, probing depth and radiographic examination. Patients were not included if they were not willing, or able to give informed consent, or if they presented with: i) pain before treatment, ii) a chronic condition requiring the intake of anti-inflammatory/antibiotic drugs, iii) a non-restorable tooth, or iv) root canals in which patency for periapical fluid sampling could not be achieved.

**Sample size estimation**

Binary/dichotomized outcomes such as residual bacterial growth and pain after the cleaning and shaping procedure typically require more than 100 cases per group to test for equality or non-inferiority (Laster *et. al*. 2006). The more sensitive outcome yielding continuous data assessed here was the change of periapical MMP-9 levels according to treatment. However, no clinical trials were identified that would allow deducing a power analysis (Wahlgren *et al.* 2002, Martinho *et al.* 2016). Hence, the number of patients was set to be 30 per group, which is a sufficient number for a first tentative statistical analysis on continuous data (Krithikadatta 2014). However, since no proper sample size estimation was performed, the *P* values reported here should be interpreted with care.

**Clinical procedures**

A CONSORT flow diagram outlining the treatment methodology is represented in Fig. 1. Sixty patients (males-35; females-25) from the age group 18-65 years meeting the inclusion criteria participated. Patients were randomly divided into two groups based on irrigation regimen. Random sequence generation was performed using a computer-generated number (www.randomizer.org), and allocation concealment was achieved using a block randomization technique (block size of six) with 1:1 allocation ratio. One researcher (JV) picked a closed envelope containing the instruction to either use the pure 2.5% NaOCl solution, or 2.5% NaOCl containing the freshly dissolved HEDP powder (Dual Rinse, Weinfelden, Switzerland). This researcher prepared 30 mL of test or control solution, and then handed it to the investigator performing the clinical procedures in an amber glass bottle.

The irrigation solutions under investigation cannot be discerned from each other, neither by smell nor colour. Consequently, both, the operator and the patient were blinded to the respective irrigant used.

Teeth in both groups were treated according to a standard protocol. Only one root canal per patient was evaluated. In case of multi-rooted teeth, the samples for both, microbial and MMP analysis, were collected from one individual root canal without an isthmus, which was clearly associated with the periapical radiolucency. Fourty-three of the canals included in this study were from single-rooted teeth, 12 were buccal canals in maxillary premolars, 2 palatal canals in maxillary molars, and 3 distal canals in mandibular molars. Canals were selected based on anatomical similarity and controllability of the sampling procedure. The teeth were anaesthetized (2% lidocaine hydrochloride with epinephrine 1:80,000; Septodont, Saint-Maur-des-Fosses, France) and isolated with rubber dam (Hygenic; Coltène Whaledent, Altstätten, Switzerland). The operating field was disinfected by swabbing with 30% hydrogen peroxide, followed by 5% tincture of iodine (Möller 1966). Subsequently, the access cavity was prepared using a sterile diamond-coated bur (Horico, Berlin, Germany), and working length was estimated using the preoperative radiograph. Patency of the root canal was achieved using size 10 K-file (Dentsply Sirona Endodontics, Ballaigues, Switzerland). Working length was determined using an electronic apex locator (Root ZX; Morita, Osaka, Japan). The canal was enlarged to size 20 using hand instrumentation. The pre-treatment microbial sample (S1) was then collected by placing a size 20 sterile paper point (Dentsply Sirona Endodontics) to working length for one min. Subsequently, the paper point was immediately placed inside a sterile centrifugation tube containing 20 mL of thioglycollate broth (Merck, Darmstadt, Germany). This procedure was repeated with a second paper point, which was added to the same broth. Then, the root canal was apically enlarged to size 30 (F3) using ProTaper Universal instruments (Dentsply Sirona Endodontics) and irrigated for the first time. Irrigation was performed using a 29-gauge side-vented needle (Vista Dental Products, Racine, WI, USA), which was kept 1 mm short of the working length. Between each instrument change, the root canal was irrigated with the allocated irrigant (5 mL for 1 min). Hence, a total of 25 mL of the irrigating solution was used. Once the shaping procedure was completed, the root canal was flushed with 5 mL of sodium thiosulfate (Merck) for 1 min, followed by 5 mL of distilled water for 1 min, to avoid potential carry-over effects by NaOCl remnants.

The root canal was dried using paper points (Dentsply Sirona Endodontics) and the post treatment microbial sample (S2) was collected as described before (see above). In addition, a

periapical fluid sample was collected by introducing a fine sterile size 20 paper point 2 mm beyond the canal terminus for 1 min (Shimauchi *et al.* 1996). This procedure was performed twice. The paper points were placed in a sterile micro-centrifugation tube (Merck) containing 2 mL of sterile physiological saline solution, and immediately transferred to a -80°C freezer until further processing. In case of multirooted teeth, the canals which were not sampled were enlarged to size #30 (F3) using ProTaper Universal instruments and were irrigated with the same group of irrigant which was used for the canal from which the samples were taken. The access cavity of the tooth was then temporized (Cavit G, 3M ESPE, Seefeld, Germany). The root canals were left empty for the interim in order to avoid any possible effects by intra-canal medicaments. All patients were recalled after one week. On the recall visit, the tooth was isolated with rubber dam and the operating field was disinfected as described above. The previously sampled root canal was re-entered, flushed with saline, and a second periapical fluid sample was collected similar to the first one (see above).

After the final sampling procedure, root canals were irrigated with 2.5% NaOCl followed by 17% EDTA (Vista Dental Products, Racine, WI, USA). Root canals were filled with AH Plus sealer and ProTaper combined with size 20 gutta-percha points (Dentsply Sirona Endodontics) using a lateral compaction technique. Subsequently, a post-endodontic restoration was placed.

**Microbiological analysis**

To test for the presence of culturable bacteria, cultures were inoculated in thioglycollate broth. For the identification of main anaerobic taxa, the root canal samples were plated on 5% sheep blood agar, neomycin blood agar and phenyl ethyl alcohol agar with metronidazole (5 µg, Oxoid, Basingstoke, UK) disc. Cultures were incubated at 37°C for 72 h in an anaerobic chamber (Whitley A35 Anaerobic workstation, Don Whitley Scientific, Shipley, UK). In addition, specimens were also cultured aerobically on 5% sheep blood agar and MacConkey's agar (BD, Becton Dickinson, Heidelberg, Germany). Bacterial morphotypes were identified employing Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF, BioMerieux, Marcy-L'Étoile, France) analysis.

**Assessment of postoperative pain**

Postoperative pain was assessed 24 h after the first visit using a numerical rating scale (NRS-11, WGMC Center, 2003), with "0" representing no pain and "10" being the worst pain

imaginable as anchors. The patients were interviewed over the telephone and their pain levels were tabulated.

### Assessment of inflammatory host response

Apparent neutrophil activity in periapical tissues was quantified using a commercially available ELISA kit targeting human MMP-9 in its pro- (92 kDa) and active (82 kDa) form (Quantikine ELISA, R&D Systems, Minneapolis, MN, USA). The kit was used according to the manufacturers recommendations. Paper points were placed in the substrate solution and agitated for one hour on a platform shaker for protein extraction. Absorbance was read at 450 nm using a microplate reader (LISA Plus, Mumbai, India). The standard curve was generated using a four-parameter logistic curve fit for each set of samples assayed.

MMP levels were normalized to total protein (TP) in each sample. TP was determined using a modification of Lowry's method (Peterson 1977) against a standard series of bovine serum albumin. Readings were taken after 30 min using the microplate reader at 540 nm.

### Data presentation and analysis

Categorical data related to the presence/absence of infection after the cleaning and shaping procedure and postoperative pain were compared between groups using Fisher's exact test. Data related to absolute MMP-9/TP levels after cleaning and shaping and at the recall visit were skewed (Shapiro-Wilk test) and are thus presented as medians and inter-quartile ranges (IQRs). These data were compared between and within (first versus second visit) groups using Wilcoxon signed-rank test. The alpha-type error for all these comparisons was set to 5%.

## Results

### Microbiology

All initial samples collected from the root canals ($N$ = 60; S1) had positive microbial growth (Table 1). Of these canals, 46 had a mixed aerobic/anaerobic infection, while 14 showed had only aerobic growth. Irrigation with pure NaOCl rendered 12 of 30 (40%) canals free of culturable microorganisms. The corresponding treatment with the NaOCl/ Dual Rinse HEDP mixture resulted in 15 of 30 canals being free of microorganisms ($P$ = 0.60). There was no apparent selection of bacterial species by either treatment, with similar taxa predominating between groups and sampling times (Tables 1, 2). *Streptococcus mitis/oralis* and

*Entereococcus faecalis* predominated among the facultative, and *Veillonella* spp. among the strictly anaerobic taxa.

**Postoperative pain**

All 60 patients responded to the postoperative pain inquiry. One of the 30 patients in the pure NaOCl group experienced moderate postoperative pain ("6" on the NRS-11), while 2 patients in the NaOCl/ Dual Rinse HEDP group experienced mild postoperative pain ("2" on the NRS-11). None of the other patients reported postoperative pain or discomfort related to the tooth that had received the cleaning and shaping procedure reported here ($P = 1.0$).

**Inflammatory host response**

At the initial treatment visit, median and IQR MMP-9 values were 118 (69, 215) pg/mg TP in the pure NaOCl group and 169 (90, 295) in the NaOCl/ Dual Rinse HEDP group ($P > 0.05$). These values dropped significantly ($P < 0.001$) when the teeth were re-accessed and sampled one week after the cleaning and shaping procedure. Pure NaOCl induced a median change in MMP-9 of 57 (-1, 124) pg/mg TP, compared to 85 (8, 166) observed with NaOCl/ Dual Rinse HEDP. This tendency of the combined irrigant to reduce MMP-9 levels in periapical tissues more than the pure NaOCl solution did not reach statistical significance either ($P = 0.25$). MMP-9/TP values dropped in all but 12 of the 60 individual cases. Cases with high initial MMP-9/TP values tended also to have higher MMP-9/TP values at the recall visit (Spearman's Rho = 0.51, $P < 0.001$).

## Discussion

The current trial revealed no difference between irrigation using a pure 2.5% NaOCl solution and a counterpart containing 9% HEDP regarding microbial reduction during the cleaning and shaping procedure, postoperative pain, or periapical levels of an enzyme related to neutrophil activity (MMP-9).

This was a first clinical study on a chemical that has not been available for endodontic usage until recently. It was not the goal to mimic a clinical scenario (i.e. effectiveness) but rather to check whether there could be any unexpected or non-wanted effects on the NaOCl solution by admixing Dual Rinse HEDP. Ths is why EDTA was not used in the pure NaOCl group, as this would have interfered with the NaOCl and also with blinding, and did not place an interim dressing, which would have obscured the assessment of the periapical marker of

inflammation. This study is further limited by the fact that only short-term treatment outcomes were evaluated. The potential advantages of using HEDP in an NaOCl irrigant, which relate to ease of usage, the possibility to save chair time, and conditioning of the root canal wall for a subsequent root filling procedure, were not investigated in this safety trial. The current results are in line with a recently performed *in vitro* study, which showed no increase in NaOCl cytotoxicity when adding the etidronate powder under investigation (Ballal *et al.* 2019). Future clinical studies should be designed as superiority trials, and EDTA should be included in the control arm, as the sequence NaOCl-EDTA (or a chelating solution that is combined with an antiseptic) represents the current gold standard in root canal irrigation (Ma *et al.* 2011, Neelakantan *et al.* 2012). However, it should be considered that the studies leading to such irrigating protocols were done on extracted teeth, and a true evidence base remains elusive.

Root canals with simple anatomy were selected because bacterial sampling from fins and isthmus areas is doubtful (Siqueira & Rôças 2009). Whether these results can be extrapolated to teeth with more complex root anatomy requires further investigation. Nevertheless, this report is first of its kind, confirming earlier *in vitro* studies that the desired effects of NaOCl are not hampered by HEDP that is freshly admixed.

Various concentrations of NaOCl have been advocated for root canal treatment. However, it has been shown that at higher concentration, NaOCl has caustic potential (Hauman & Love 2003). Hence, in the present study 2.5% of NaOCl was used. The antibacterial effect of HEDP/NaOCl combinations against endodontic pathogens is well demonstrated in previous *in vitro* studies (Arias-Moliz *et al.* 2014, 2015). However, hitherto there has been no clinical trial evaluating possible influences that cannot be simulated *in vitro*. In the present study, irrigation with the combination of HEDP in 2.5% NaOCl irrigant caused the absence of culturable microorganisms in 50% of the root canals under investigation, compared to 40% when 2.5% NaOCl was used alone. This result is in accordance with previous clinical observations, which have shown an incidence of negative cultures after irrigation with NaOCl ranging from 40%-60% (Byström & Sundqvist 1985, Siqueira *et al.* 2007). This result clearly shows that in clinics, the antibacterial effect of NaOCl is not hampered by HEDP when both reagents are freshly combined.

The microorganisms that were identified in the root canals of teeth presenting with primary non-symptomatic apical periodontitis in the current study are in line with published material. *S. mitis* and *Veillonella* spp., and *F. nucleatum* are consistently found as main taxa in such cases (Hommez *et al.* 2004, Rôças & Siqueira 2018). However, 13 of the 60 initial root canal

samples (S1) had only aerobic growth, which may reflect the difficulty in recovering and culturing strict anaerobes. The relatively high occurrence of *E. faecalis* in the present study may be related to food and oral hygiene in the studied population (Zehnder & Guggenheim 2009). In line with earlier reports, there was no apparent selection of any "hard to eliminate" taxa such as *E. faecalis* observed in the current study (Zandi *et al.* 2016).

Patients may experience discomfort and pain following root canal treatment (Pak & White 2011). Several variables are associated with this outcome, including factors related to root canal irrigation (da Silva *et al.* 2015). Various methods are described to evaluate the intensity of postoperative pain (Ferreira-Valente *et al.* 2011). In this study, a numeric pain rating scale was used for evaluation (Warren Grant Magnusson Clinical Center 2003). The reason for this was that the scale could be enquired in a telephone interview. Preoperative pain is a main predictor for the presence of pain after root canal treatment (Law *et al.* 2015). Therefore, patients presenting without pain were selected for this trial. Postoperative pain occurred rarely (1.8 % of the patients, $n = 3$). Hence, an influence on postoperative pain of the irrigants used here can be regarded as negligible under the current conditions. This result is in accordance with a previous study, which reported a low postoperative pain incidence and, if occurring, merely moderate pain levels after single-visit root canal treatment of teeth with asymptomatic apical periodontitis employing different irrigants (Almeida *et al.* 2012).

Analysis of periapical fluid was used to assess change in the levels of the neutrophil-derived gelatinase MMP-9. As has been shown in previous investigations, the change of neutrophil enzyme levels in periapical fluid between the first and the second visit can provide information regarding the healing propensity of periapical tissues (Wahlgren *et al.* 2002, Alptekin *et al.* 2005). In the present study, MMP-9 levels in periapical fluid declined significantly in one week without significant differences between treatment groups. This reduction in the MMP-9 levels may be attributed to the antibacterial activity of the irrigants, as was confirmed in the microbiological analysis. However, there was no correlation between MMP-9 levels and the presence/absence of culturable bacteria in the main root canal. This may be related to a known shortcoming of bacterial culture from the root canal: samples are taken from the entire canal rather than the apical one third, which is the clinically relevant aspect (Baumgartner & Falkler 1991).

Further clinical trials should assess the influence of HEDP in a NaOCl irrigant on disinfection in teeth with complex anatomies (i.e. molar teeth). These studies could be performed using molecular microbiological methods to compare the antibacterial effect of Dual Rinse HEDP and NaOCl irrigants on continuous data and thus gain statistical power.

Furthermore, long-term follow-up of patients is required to observe the radiographic healing of chronic apical periodontitis over time. Moreover, the correlation between the change in neutrophil marker levels in inflamed periapical tissues one week after treatment and the long-term healing of the lesions associated with these teeth should be investigated.

## Conclusions

Under the conditions of the current study, no adverse effects of adding an etidronate (Dual Rinse HEDP) to a 2.5% NaOCl solution were detected. The antimicrobial effect of the NaOCl was maintained, whilst no apparent inflammatory effects to the periapical tissues in the form of post-operative pain or an increase in a marker molecule related to neutrophil activity (MMP-9) were added.

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Matthias Zehnder reports a conflict of interest in the form of a patent application (EP3284456A; US 20180042821) licensed to Medcem llc. The other authors deny any conflict of interest. The trial was approved by the institutional ethics committee (Kasturba Hospital, Manipal, IEC 135/2017) and registered at Clinical Trials Registry - India (CTRI/2017/08/009493 - 25/08/2017).

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**Figure legends**

**Figure 1** The Consolidated Standards of Reporting Trials (CONSORT) flow diagram of patients included in this study.

**Table 1** Microbiological Results at S1 (before Instrumentation/Irrigation)

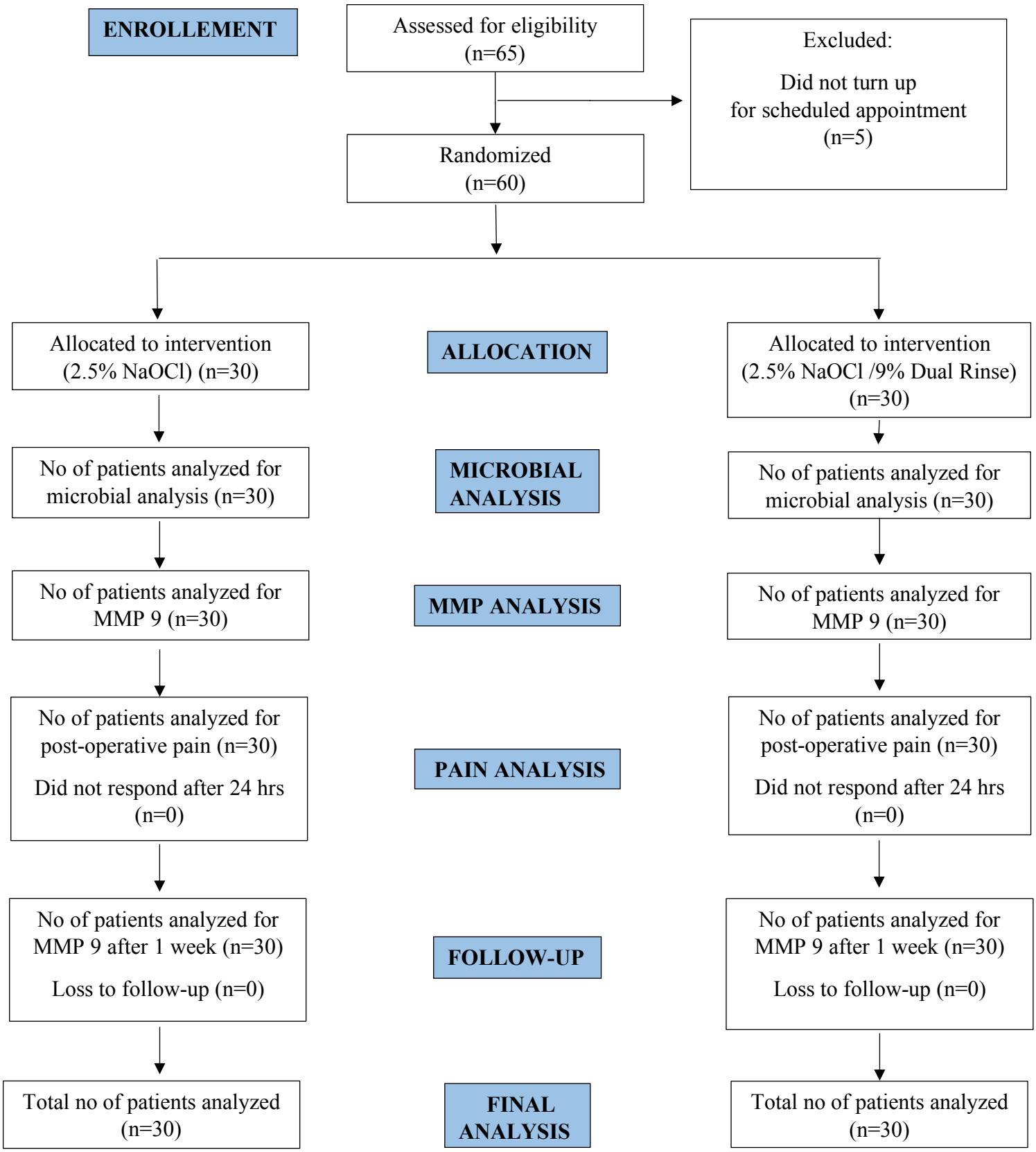
| **Group** | **Growth/no growth** | **Main species* (frequency of identification)** |
|---|---|---|
| 2.5% NaOCl purum | Aerobic: 30/0 | *Streptococcus mitis/oralis* (21)<br>*Enterococcus faecalis* (6)<br>*Streptococcus anginosus* (2)<br>*Neisseria mucosa* (1)<br>*Staphylococcus epidermidis* (1)<br>*Staphylococcus hominis* (2)<br>*Staphylococcus lugdunensis* (1)<br>*Streptococcus constellatus* (1)<br>*Streptococcus gordonii* (1)<br>*Streptococcus parasanguinis* (1) |
| | Anaerobic: 24/6 | *Veillonella atypica* (9)<br>*Veillonella parvula* (9)<br>*Fusobacterium nucleatum* (5)<br>*Bifidobacterium* spp (1) |
| 2.5% NaOCl / 9% DR HEDP | Aerobic: 30/0 | *Streptococcus mitis/oralis* (13)<br>*Enterococcus faecalis* (10)<br>*Streptococcus parasanguinis* (4)<br>*Streptococcus gordonii* (2)<br>*Staphylococcus hominis* (2)<br>*Bacillus cereus* (1)<br>*Klebsiella pneumoniae* (1)<br>*Staphylococcus epidermidis* (1)<br>*Staphylococcus hominis* (1)<br>*Streptococcus anginosus* (1)<br>*Streptococcus constellatus* (1) |
| | Anaerobic: 23/7 | *Veillonella atypica* (12)<br>*Veillonella parvula* (9)<br>*Fusobacterium nucleatum* (3)<br>*Bifidobacterium* spp (1)<br>*Parvimonas micra* (1)<br>*Prevotella buccae* (1) |

* In growth-positive samples, the 1-2 main aerobic and anaerobic species were identified by MALDI-TOF.

**Table 2** Microbiological Results at S2 (after Instrumentation/Irrigation)

| Group | Growth/no growth | Main species* (frequency of identification) |
|---|---|---|
| 2.5% NaOCl purum | Aerobic: 18/12 | *Streptococcus mitis/oralis* (12)<br>*Enterococcus faecalis* (3)<br>*Staphylococcus epidermidis* (2)<br>*Staphylococcus hominis* (2)<br>*Bacillus cereus* (1)<br>*Neisseria mucosa* (1)<br>*Streptococcus parasanguinis* (1)<br>*Streptococcus constellatus* (1) |
| | Anaerobic: 6/24 | *Veillonella parvula* (4)<br>*Fusobacterium nucleatum* (2)<br>*Veillonella atypica* (1) |
| 2.5% NaOCl / 9% DR HEDP | Aerobic: 15/15 | *Streptococcus mitis/oralis* (10)<br>*Enterococcus faecalis* (4)<br>*Bacillus cereus* (1)<br>*Neisseria mucosa* (1)<br>*Streptococcus parasanguinis* (1)<br>*Streptococcus constellatus* (1) |
| | Anaerobic: 6/24 | *Veillonella parvula* (3)<br>*Veillonella atypica* (2)<br>*Fusobacterium nucleatum* (1) |

* In growth-positive samples, the 1-2 main aerobic and anaerobic species were identified by MALDI-TOF.


ENROLLEMENT
Assessed for eligibility (n=65)
Excluded:
Did not turn up for scheduled appointment (n=5)
Randomized (n=60)
Allocated to intervention (2.5% NaOCl) (n=30)
ALLOCATION
Allocated to intervention (2.5% NaOCl /9% Dual Rinse) (n=30)
No of patients analyzed for microbial analysis (n=30)
MICROBIAL ANALYSIS
No of patients analyzed for microbial analysis (n=30)
No of patients analyzed for MMP 9 (n=30)
MMP ANALYSIS
No of patients analyzed for MMP 9 (n=30)
No of patients analyzed for post-operative pain (n=30)
Did not respond after 24 hrs (n=0)
PAIN ANALYSIS
No of patients analyzed for post-operative pain (n=30)
Did not respond after 24 hrs (n=0)
No of patients analyzed for MMP 9 after 1 week (n=30)
Loss to follow-up (n=0)
FOLLOW-UP
No of patients analyzed for MMP 9 after 1 week (n=30)
Loss to follow-up (n=0)
Total no of patients analyzed (n=30)
FINAL ANALYSIS
Total no of patients analyzed (n=30)